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GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER 1/1  
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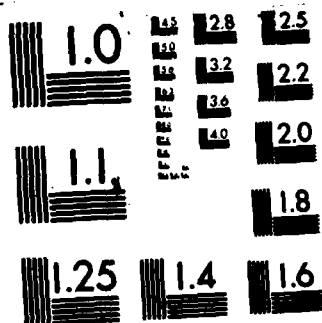
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GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER  
GROUP OF VIRUSES

ANNUAL REPORT

DR. DAVID H.L. BISHOP  
AUGUST 1979

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University of Alabama in Birmingham  
Birmingham, Alabama 35294

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) <b>The reporting period represents 18 months since the inception of the project. During this time we have (1) characterized several of the major structural components of the Phlebotomus fever group viruses (Robeson, et al, 1979) and (2) isolated and begun to characterize several temperature sensitive (ts), conditional lethal, mutants of Punta Toro (PT) virus. In addition at the request of WRAIR, we have (3) demonstrated the feasibility of using oligonucleotide fingerprinting to distinguish Dengue (DEN) virus serotypes using</b>  <b>continued on back...</b>		

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(1) Analyses of the major structural components of the Phlebotomus fever viruses have established that Karimabad (KAR), PT, Chagres (CHE), Candiru (CDU), Itaporanga (ITP) and the Sicilian and Naples sandfly fever (SFS, SFN) viruses, each has a tripartite RNA genome and three major structural polypeptides (two glycoproteins, G1 and G2, and an internal, nucleocapsid associated, protein N). Both the mol. wt. of the major structural polypeptides and the virion RNA segments of the different Phlebotomus fever viruses are easily distinguished from those of the California encephalitis serogroup viruses and to various extents (depending on the virus) from each other. Tryptic peptide analyses of  $^{35}\text{S}$  and  $^3\text{H}$  methionine labeled G1 and G2 polypeptides of KAR have established that these two polypeptides have distinguishable sequences. The behaviour of reduced KAR G1 and G2 polypeptides on polyacrylamide gel electrophoresis is aberrant by comparison with unreduced preparations.

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(3) Oligonucleotide fingerprint analyses of prototype DEN 1, 2, 3 and 4 viruses involving both single and mixed coelectropherograms of ribonuclease T1 digests of  $^{32}\text{P}$  labeled 40S viral RNA samples have shown that each has a unique fingerprint that is easily distinguished from that of another prototype DEN virus. Evidence has been obtained indicating that the 5' sequence of DEN 2 RNA is m7GpppAmpXp...



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## I. SUMMARY

The reporting period represents 18 months since the inception of the project. During this time we have (1) characterized several of the major structural components of the Phlebotomus fever group viruses (Robeson, et al, 1979) and (2) isolated and begun to characterize several temperature sensitive (ts), conditional lethal, mutants of Punta Toro (PT) virus. In addition at the request of WRAIR, we have (3) demonstrated the feasibility of using oligonucleotide fingerprinting to distinguish Dengue (DEN) virus serotypes using cloned *Aedes albopictus* cells to produce labeled virus. These procedures have now been adopted by WRAIR personnel for their Dengue virus studies.

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## II. REPORT

### A. Introduction.

The objectives of this contract are to determine the genetic capacity of the Phlebotomus fever group viruses and the implications of forming new Phlebotomus fever virus genotypes. Since members of this group of exotic viruses cause illnesses in epidemic proportions in different parts of the world, and therefore are of military significance, our objectives relate to the question of deriving vaccine strains of viruses which will be useful in protecting military and civilian personnel against virus infections.

To realize these objectives we aim to develop genetic tools in the form of temperature sensitive (ts), conditional lethal, mutants of particular member viruses, and use these in mixed virus infections to produce new virus genotypes presumably by RNA segment reassortment (Gentsch & Bishop, 1976; Gentsch, et al., 1977b). By knowing the RNA segment coding assignments (Gentsch & Bishop, 1978, 1979), we will be able to produce custom genotypes of certain Phlebotomus fever group viruses containing particular genetic information and gene products. Such genotypes will eventually be tested for their pathogenicity and vaccine capabilities in model animal systems and compared to the prototype strains. If effective, then similar procedures will be used to derive reassortant viruses in clean cell systems in order to obtain virus vaccines that can be used to immunize man.

This report therefore describes both analyses of the genetic potential of selected Phlebotomus fever group viruses, and initial molecular studies to characterize the viral genomic RNA species and their gene products.

In addition, at the request of WRAIR, we have employed one of the biochemical procedures commonly now used to differentiate virus isolates (namely oligonucleotide fingerprinting) to determine the feasibility of differentiating the 4 DEN prototype viruses. The results of these studies are also given in this report.

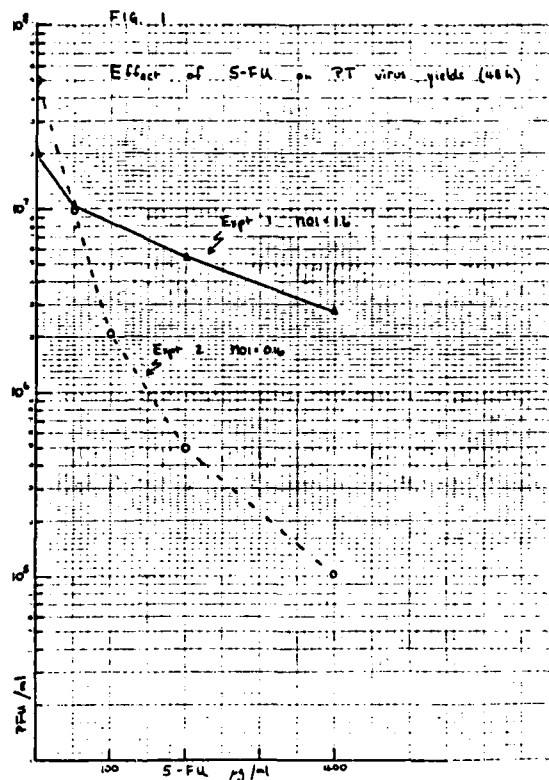
### I. Genetic studies.

We received from WRAIR cloned virus samples of the following viruses belonging to the Phlebotomus fever serogroup: CDU, CHG, ITP, KAR, SFN, PT, and two isolates of the Sicilian sandfly fever virus. In addition we have received from Dr. R. Shope (YARU), Icoaraci (ICO) virus, as well as other stocks of the above viruses.

Each virus has been recloned and passaged at 35°C in Vero cells to produce working stocks, giving titers in Vero cells at 35°C ranging from  $4.3 \times 10^6$  (Naples sandfly fever virus) to  $5.3 \times 10^6$  (Punta Toro).

In initial studies with these viruses, for each virus similar numbers of plaques were obtained at 35°C and 38°C in Vero cells (staining on days 5-7). However at 39.8°C there were either no plaques or less than 1% of those obtained at 35°C (depending on the virus). We have been successful at high temperature adaptation through serial passaging of PT virus to 39.8°C giving a 39.8°C/35°C E.O.P. of 0.8. Similar attempts to adapt certain of the other Phlebotomus fever group viruses are in progress. The plaque assay commonly adopted for titrating Phlebotomus fever group viruses involves a 0.9% agar overlay containing Medium 199, 10% (v/v) heat inactivated MEM, fetal calf serum, 0.75% DMSO, and additional vitamins and essential amino acids.

Using PT virus, *ts* mutants have been selected by standard procedures (Gentsch & Bishop, 1976) and from 500 clones of progeny grown in the presence of 50  $\mu$ g of 5-fluorouracil (see Fig.1), some 24 *ts* mutant have been obtained.



Working stocks have been derived for the 24 *ts* mutants and titered at 35°C (permissive temperature) and 39.8°C (nonpermissive temperature) (Table I). Fig. 1. Effect of 5 FU on PT virus yields as a function of mutagen dose and M.O.I.

TABLE I: TITERS OF PUNTA TORO VIRUS *ts* MUTANTS

Mutant	35°C	39.8°C	E.O.P. 39.8°C/35°C
<i>ts</i> 1	4.6x10 <sup>7</sup>	7.3x10 <sup>5</sup>	1.3x10 <sup>-2</sup>
<i>ts</i> 2	9.6x10 <sup>6</sup>	< 10 <sup>5</sup>	< 1.0x10 <sup>-2</sup>
<i>ts</i> 3	1.9x10 <sup>7</sup>	< 10 <sup>5</sup>	< 5.2x10 <sup>-3</sup>
<i>ts</i> 4	2.0x10 <sup>7</sup>	2.0x10 <sup>5</sup>	1.0x10 <sup>-2</sup>
<i>ts</i> 5	2.2x10 <sup>7</sup>	< 10 <sup>5</sup>	< 4.5x10 <sup>-3</sup>
<i>ts</i> 6	3.8x10 <sup>7</sup>	< 10 <sup>5</sup>	< 2.6x10 <sup>-4</sup>
<i>ts</i> 7	5.3x10 <sup>7</sup>	< 10 <sup>5</sup>	< 1.9x10 <sup>-4</sup>
<i>ts</i> 8	1.4x10 <sup>7</sup>	< 10 <sup>5</sup>	< 7.1x10 <sup>-5</sup>
<i>ts</i> 9	1.8x10 <sup>7</sup>	1.0x10 <sup>5</sup>	5.3x10 <sup>-5</sup>
<i>ts</i> 10	2.0x10 <sup>8</sup>	< 10 <sup>5</sup>	< 5.0x10 <sup>-4</sup>
<i>ts</i> 11	4.0x10 <sup>5</sup>	2.0x10 <sup>3</sup>	5.0x10 <sup>-3</sup>
<i>ts</i> 12	5.4x10 <sup>6</sup>	< 10 <sup>5</sup>	< 1.7x10 <sup>-4</sup>
<i>ts</i> 13	4.0x10 <sup>6</sup>	1.0x10 <sup>3</sup>	2.5x10 <sup>-4</sup>
<i>ts</i> 14	2.3x10 <sup>6</sup>	2.0x10 <sup>3</sup>	8.6x10 <sup>-4</sup>
<i>ts</i> 15	1.2x10 <sup>7</sup>	< 10 <sup>5</sup>	< 8.3x10 <sup>-5</sup>
<i>ts</i> 16	7.0x10 <sup>6</sup>	< 10 <sup>5</sup>	< 1.4x10 <sup>-4</sup>
<i>ts</i> 17	1.0x10 <sup>7</sup>	< 10 <sup>5</sup>	< 5.3x10 <sup>-5</sup>
<i>ts</i> 18	3.0x10 <sup>6</sup>	< 10 <sup>5</sup>	< 3.3x10 <sup>-4</sup>
<i>ts</i> 19	1.2x10 <sup>7</sup>	< 10 <sup>5</sup>	< 8.3x10 <sup>-5</sup>
<i>ts</i> 20	2.0x10 <sup>6</sup>	< 10 <sup>5</sup>	< 5.0x10 <sup>-4</sup>
<i>ts</i> 21	2.0x10 <sup>6</sup>	< 10 <sup>5</sup>	< 5.0x10 <sup>-4</sup>
<i>ts</i> 22	8.4x10 <sup>6</sup>	< 10 <sup>5</sup>	< 1.1x10 <sup>-4</sup>
<i>ts</i> 23	8.0x10 <sup>6</sup>	< 10 <sup>5</sup>	< 1.2x10 <sup>-4</sup>
<i>ts</i> 24	4.4x10 <sup>6</sup>	< 10 <sup>5</sup>	< 2.2x10 <sup>-4</sup>
wild-type	1.1x10 <sup>7</sup>	8.3x10 <sup>6</sup>	0.77



Recombination assays have been initiated with five PT ts mutants (Table 2), although no evidence for recombination has yet been obtained. This result is not too surprising since for the California encephalitis serogroup virus, Tahyna (TAH), 20 of 21 TAH ts viruses were found belonging to one recombination group with the 21st TAH ts virus isolate belonging to a second group (E. Rozhon and D.H.L. Bishop, unpublished data). Further mutant virus isolations and recombination experiments with PT ts mutant are in progress.

TABLE 2: VIRUS YIELDS AFTER SINGLE AND MIXED INFECTIONS  
WITH CERTAIN PUNTA TORO VIRUS ts MUTANTS

Inoculum	Incubation temp (°C)	48 h yield assayed at:		% Recombinants*
		35°C	39.8°C	
<u>ts</u> 2	35	5.3x10 <sup>5</sup>	< 10 <sup>3</sup>	-
<u>ts</u> 3	35	4.7x10 <sup>5</sup>	< 10 <sup>3</sup>	-
<u>ts</u> 5	35	5.8x10 <sup>5</sup>	< 10 <sup>3</sup>	-
<u>ts</u> 6	35	9.9x10 <sup>5</sup>	< 10 <sup>3</sup>	-
<u>ts</u> 7	35	2.1x10 <sup>7</sup>	< 10 <sup>3</sup>	-
<u>ts</u> 2 x <u>ts</u> 3	35	5.4x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 2 x <u>ts</u> 5	35	9.6x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 2 x <u>ts</u> 6	35	6.6x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 2 x <u>ts</u> 7	35	6.3x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 3 x <u>ts</u> 5	35	9.8x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 3 x <u>ts</u> 6	35	8.7x10 <sup>5</sup>	< 10 <sup>3</sup>	0
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<u>ts</u> 5 x <u>ts</u> 6	35	8.7x10 <sup>5</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 5 x <u>ts</u> 7	35	7.0x10 <sup>7</sup>	< 10 <sup>3</sup>	0
<u>ts</u> 6 x <u>ts</u> 7	35	8.4x10 <sup>5</sup>	< 10 <sup>3</sup>	0
wild-type	35	1.1x10 <sup>7</sup>	8.3x10 <sup>5</sup>	-

\*Percentage of recombinants =

$$\frac{[(AB)_{35/39.8} - (A_{35})_{39.8} - (B_{35})_{39.8}]}{(AB)_{35/35}} \times 100 \times 2$$

## 2. Biochemical studies of the Phlebotomus fever group viruses.

Analyses of the virion polypeptides and genomic RNA species of the Phlebotomus fever group viruses (Bishop, et al., 1978; Robeson et al., 1979; Bishop, 1979a, b) have been directed towards determining whether these viruses structurally parallel members of the Bunyavirus genus of bunyaviruses by having tripartite genomes and three major virion polypeptides. Since serologic evidence has indicated that there is no detectable serologic homology between members of the Bunyavirus genus and the Phlebotomus fever group viruses, we have been interested in determining if a rationale can be developed for forming a new genus of bunyaviruses based on the unique structural and serologic properties of the Phlebotomus fever group members. Thirdly these analyses have been undertaken as the prelude to determining if recombination involving RNA segment reassortment occurs and which viral RNA species codes for which virion polypeptide.

# a. RNA analyses.

Sucrose gradient centrifugation (Fig. 2, PT, CHG, SFS and KAR viruses), and polyacrylamide gel electrophoresis analyses (Fig. 3, 4, PT and KAR viruses) of RNA extracted from certain *Phlebotomus* fever group viruses have demonstrated that representative viruses of the group have tripartite RNA genomes, designated large (L), medium (M) and small (S).

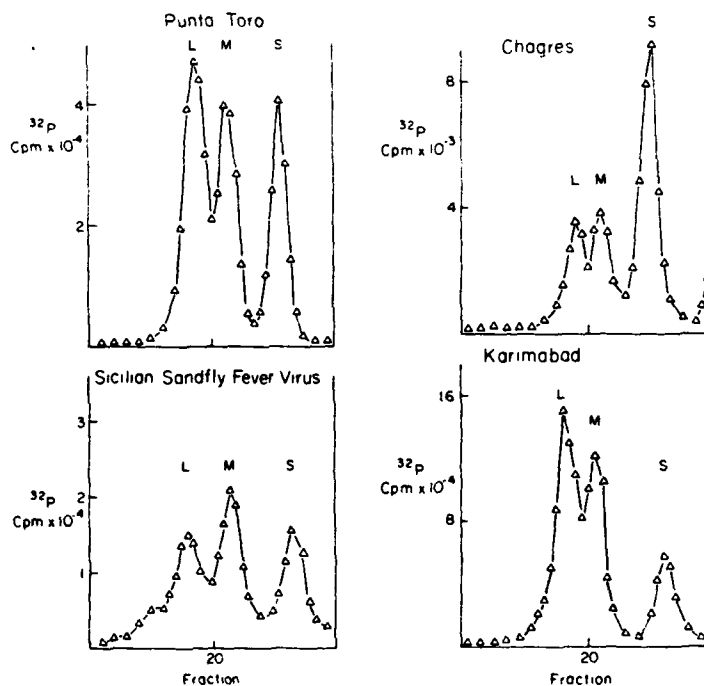


FIG. 2. Sucrose gradient resolution of the viral RNA species of Punta Toro, Sicilian sandfly fever, Chagres, and Karimabad viruses. The  $^{32}\text{P}$ -labeled viral RNA species were resolved by SDS-sucrose gradient centrifugation as described previously.

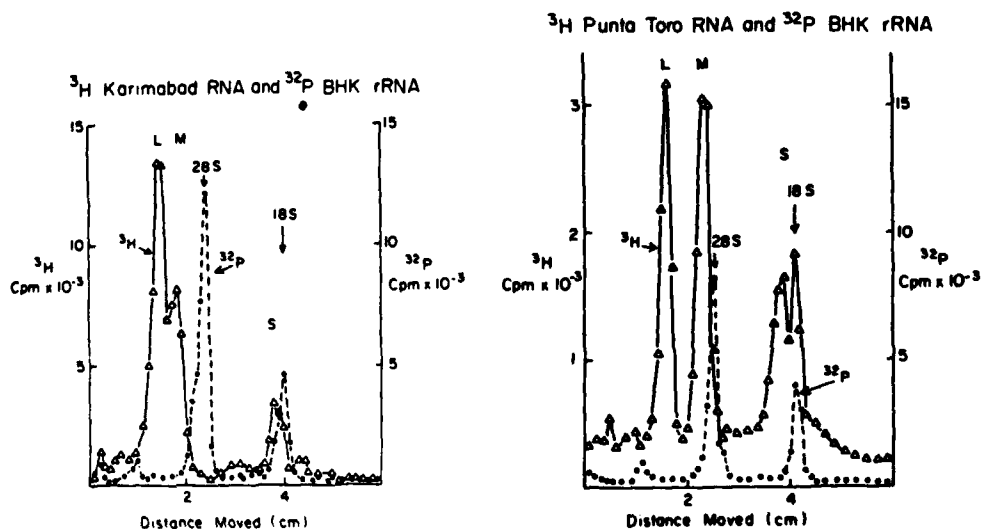


FIG. 3. Resolution of the viral RNA species of Karimabad and Punta Toro viral RNA species by polyacrylamide gel electrophoresis with marker BHK-21 cell 28S and 18S rRNA species. Samples of [ $^3\text{H}$ ]-uridine-labeled viral RNA species with  $^{32}\text{P}$ -labeled cell rRNA species were resolved by 2.4% polyacrylamide gel electrophoresis as described previously.

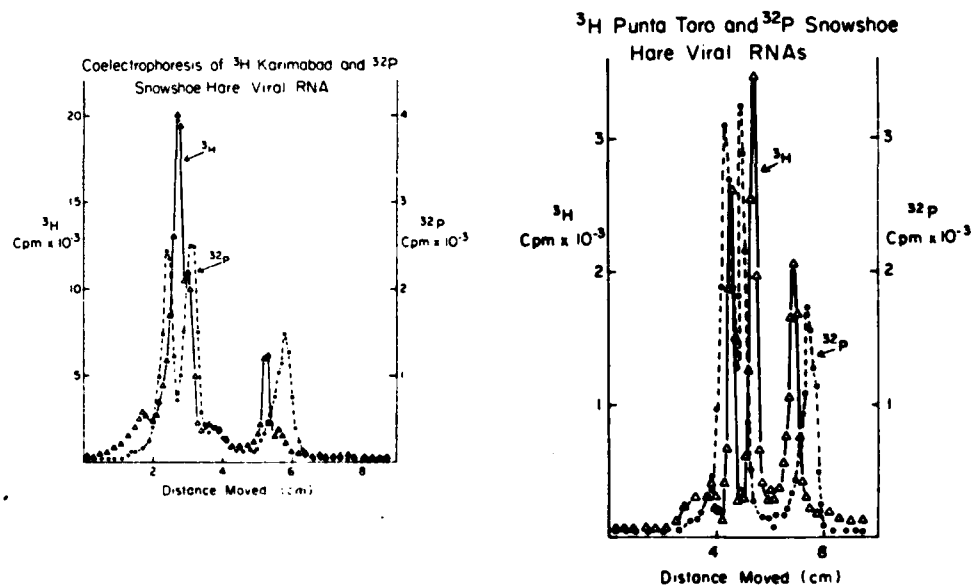


FIG. 4. Resolution of the viral RNA species of Karimabad and snowshoe hare viruses and of Punta Toro and snowshoe hare viruses by polyacrylamide gel electrophoresis. Samples of [ $^3\text{H}$ ]uridine-labeled *Phlebotomus* fever group viral RNA species and  $^{32}\text{P}$  labeled snowshoe hare virus RNA were resolved by 2.4% polyacrylamide gel electrophoresis as described previously.

Similar results involving sucrose gradient RNA analyses have been obtained for all eight *Phlebotomus* fever group viruses on hand, and with respect to electrophoretic resolution, for CHG, CDU, and the Sicilian and Naples sandfly fever viruses.

For KAR, PT (Fig. 5), Chagres, and the Sicilian and Naples sandfly fever viruses, oligonucleotide fingerprint analyses of their individual L, M and S RNA species have demonstrated that each has a unique RNA sequence, specific to the virus isolate.

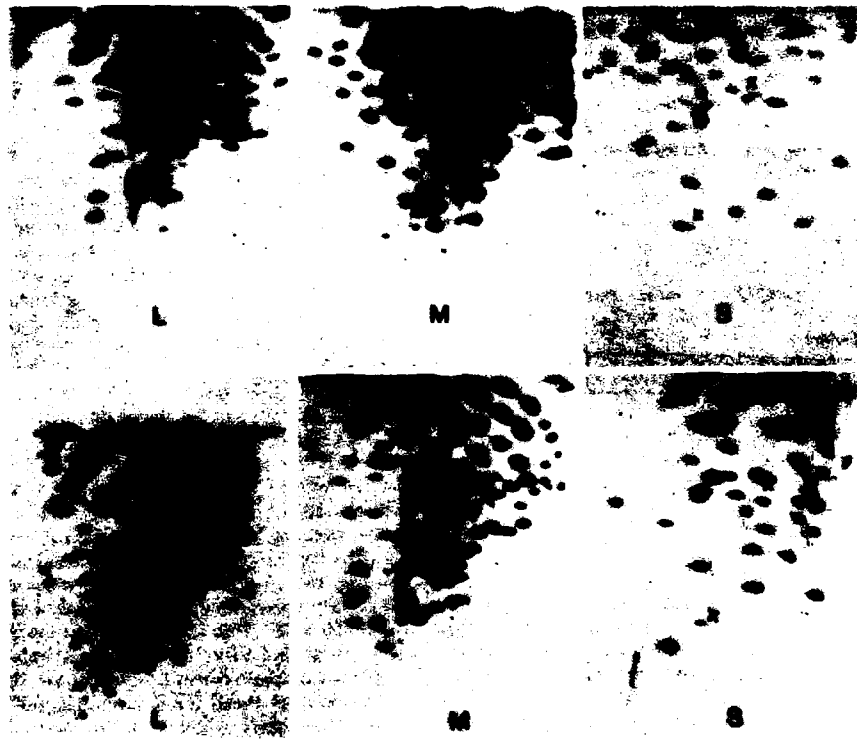


FIG. 5. Oligonucleotide fingerprint analyses of the L, M, and S RNA species of Karimabad virus (lower three panels) and Punta Toro virus (upper three panels). The oligonucleotide fingerprints of  $^{32}\text{P}$ -labeled viral RNA species were obtained as described in the text and elsewhere (10).

b. Viral polypeptide.

The major virion polypeptides of KAR, PT, SFS and CHG viruses have been analysed by polyacrylamide gel electrophoresis (see Fig. 6, 7). From the results obtained with reduced samples two or three major virion polypeptides were identified for each virus.

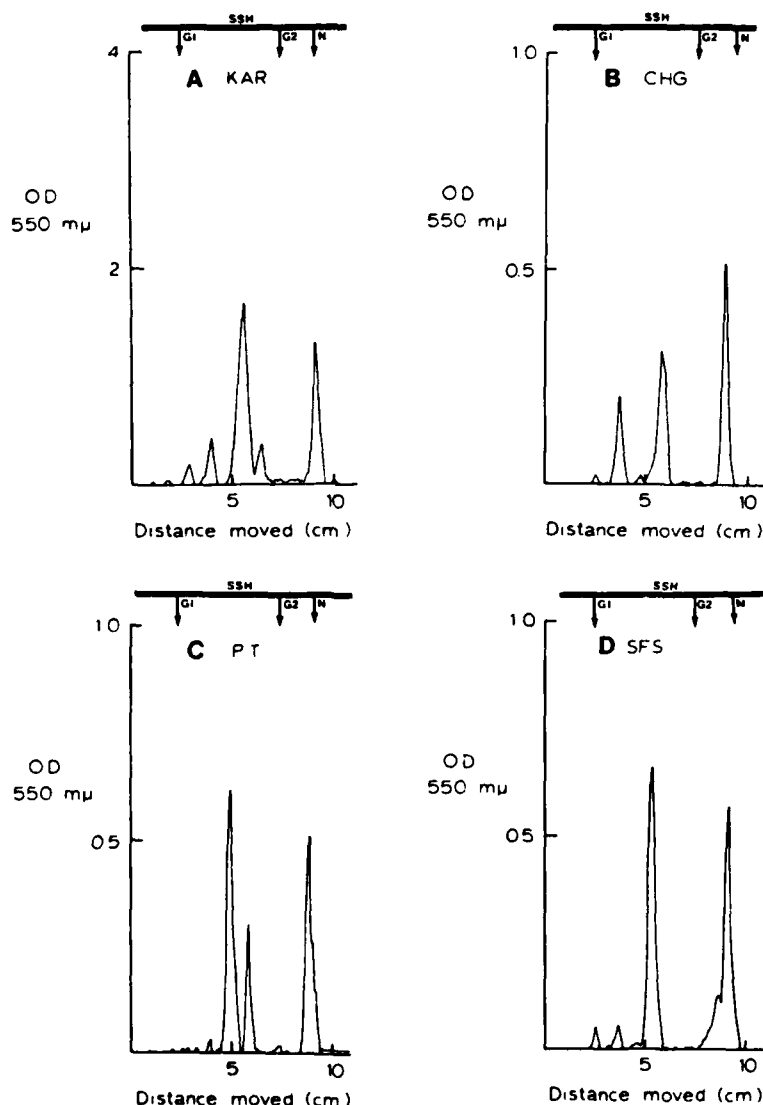


FIG. 6. Polyacrylamide gel electrophoresis of the virion polypeptides of (A) Karimabad, (B) Chagres, (C) Punta Toro, and (D) Sicilian sandfly fever viruses. Preparations of [ $^3$ H]leucine-labeled Phlebotomus fever group and snowshoe hare viruses were dissociated with SDS in the presence of 1%  $\beta$ -mercaptoethanol and were resolved by discontinuous slab polyacrylamide gel electrophoresis. After fluorography, the samples were scanned at 550 nm with a Schoeffel spectrodensitometer. The comparable positions of the snowshoe hare G1, G2, and N viral polypeptides are indicated in the scans of the Phlebotomus fever group virion polypeptides. For these Phlebotomus fever group virus preparations, successive glycerol tartrate and sucrose gradient centrifugation was used followed by virus pelleting.

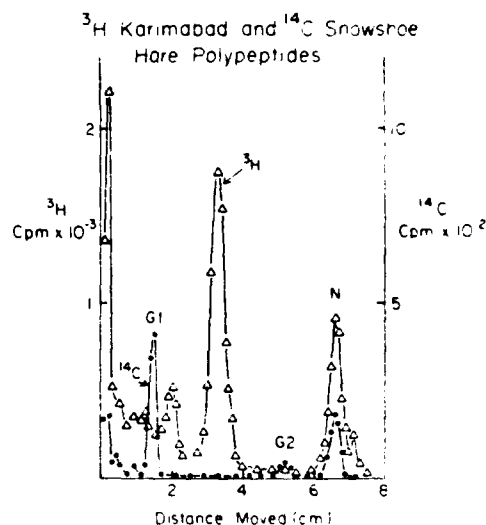


FIG. 7. Coelectrophoresis of [ $^3\text{H}$ ]leucine labeled Karimabad virus and [ $^{14}\text{C}$ ]amino acid labeled snowshoe hare virus on continuous polyacrylamide gels. Samples of the two viruses were purified as described in Fig. 6, dissociated by SDS in the presence of 1%  $\beta$ -mercaptoethanol, and resolved by polyacrylamide gel electrophoresis. The identities of the three major snowshoe hare virus polypeptides, G1, G2, and N are indicated.

Since we encountered problems in maintaining the integrity of virus particles by successive gradient centrifugation and pelleting, an alternative approach involving glycerol tartrate centrifugation and dialysis has been employed giving preparations of virus with mostly intact particles (Robeson *et al.*, 1979). For KAR virus prepared this way the virion polypeptides of reduced (Fig. 8A) or unreduced samples (Fig. 8B) have been compared using [ $^{14}\text{C}$ ]amino acid and [ $^3\text{H}$ ]glucosamine labeled virus preparation.

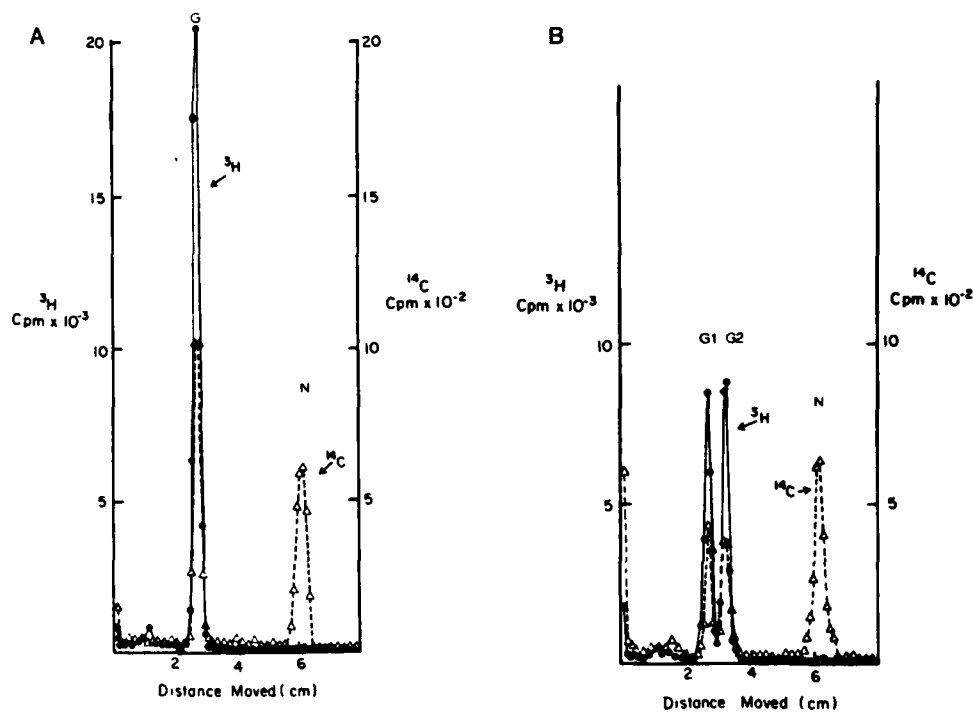


FIG. 8. Effect of  $\beta$ -mercaptoethanol on Karimabad virus polypeptides and identification of Karimabad virus glycoproteins. Preparations of [ $^3\text{H}$ ]glucosamine and [ $^{14}\text{C}$ ]amino acid-labeled Karimabad virus, purified by two successive 90-min cycles of glycerol-potassium tartrate centrifugation, followed by dialysis (see Materials and Methods), was dissociated by SDS in the presence (A) or absence (B) of  $\beta$ -mercaptoethanol. After electrophoresis, the positions and identity of the viral polypeptides were determined.

It is noteworthy for the reduced KAR virus sample shown in Fig. 8A that the profile has less high molecular weight material (aggregates?) than that found in the preparations shown in Fig. 6, 7. Also in the reduced virus preparation (Fig. 8A) only one glycoprotein size class was evident while in the unreduced preparation (Fig. 8B), two (G1 and G2) were observed. To determine whether the G1 and G2 polypeptides had distinguishable or identical sequences, preparations of  $^3\text{H}$  and  $^{35}\text{S}$  methionine labeled unreduced KAR virus were obtained and the individual G1 and G2 polypeptides recovered (Gentsch and Bishop, 1978). Mixtures of  $^3\text{H}$  G1 and  $^{35}\text{S}$  G2 (unreduced, Fig. 9A),  $^{35}\text{S}$  G1 and  $^3\text{H}$  G2 (unreduced, Fig. 9B) or  $^{35}\text{S}$  G1 and  $^3\text{H}$  G2 (reduced, Fig. 9C) were then made and resolved by polyacrylamide gel electrophoresis.

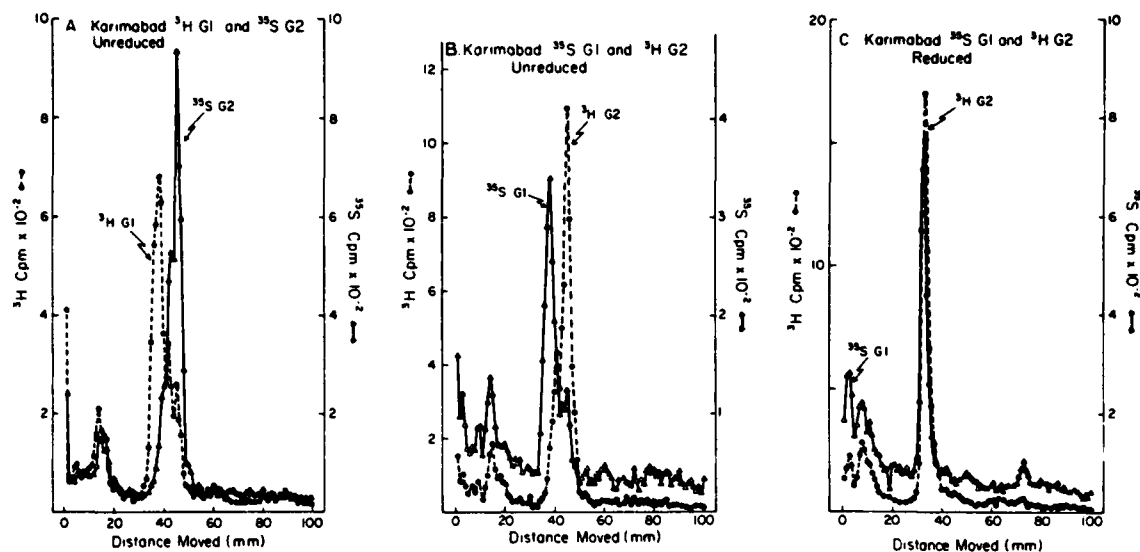


Fig. 9. Resolution of mixtures of KAR G1 and G2 polypeptides, unreduced (AB) or reduced (C) prior to and during electrophoresis.

Similar mixtures of KAR <sup>35</sup>S G1 or <sup>3</sup>H G2 and <sup>3</sup>H G1 and <sup>35</sup>S G2 polypeptides were digested with TPCK-trypsin and the products resolved by ion exchange column chromatography (Gentsch and Bishop, 1978, 1979) demonstrating that G1 and G2 are unique viral polypeptides (Fig. 10).



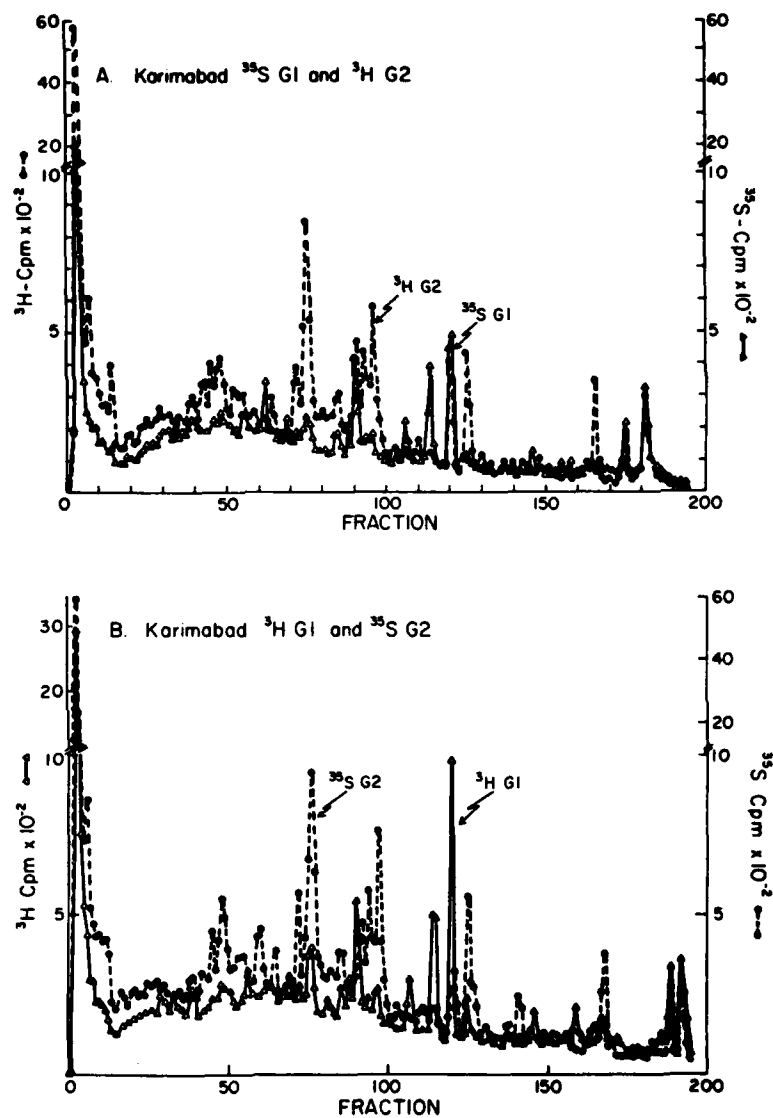


Fig. 10. Tryptic peptide analyses of TPCK-trypsin digests of (A) KAR <sup>35</sup>S G1 and <sup>3</sup>H G2 and (B) KAR <sup>3</sup>H G1 and <sup>35</sup>S G2 polypeptides.

Analyses of the location of KAR virg polypeptides involving treatment of  $^{14}\text{C}$ -amino acid of  $^3\text{H}$ -glucosamine labeled preparations with chymotrypsin to remove external surface polypeptides (Fig. 11) have established that the G1 and G2 polypeptides are susceptible to removal (i.e. external). Such treatment produces spikeless particles (Robeson *et al.*, 1979) in which the major non-glycosylated polypeptide (N) is protected from protease removal. When Triton disrupted KAR virus preparations were centrifuged to equilibrium in gradients of cesium chloride, nucleocapsids containing RNA and only N polypeptides were recovered.

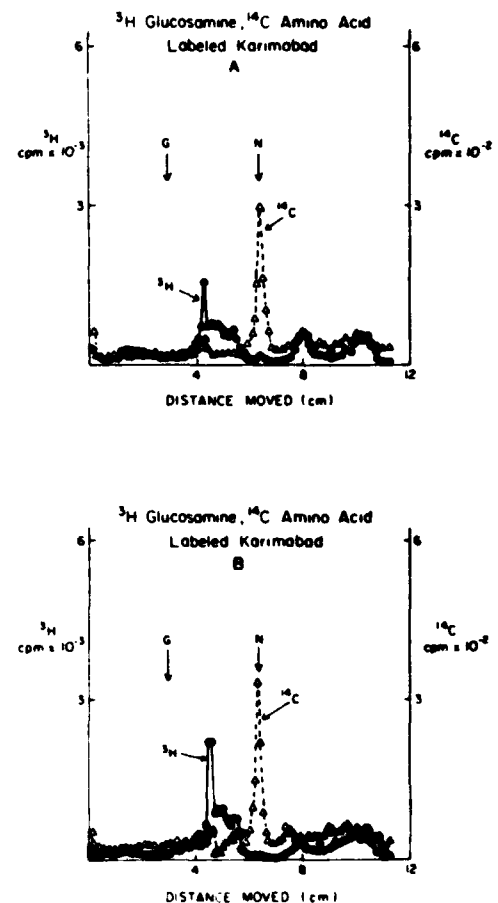


FIG. 11. Effect of  $\alpha$ -chymotrypsin on the viral polypeptides of Karimabad virus. Preparations of [ $^3\text{H}$ ]glucosamine and [ $^{14}\text{C}$ ]amino acid-labeled Karimabad virus (Fig. 6), were treated with  $\alpha$ -chymotrypsin at (A) 0.125 mg/ml versus (B) 0.300 mg/ml (final concentration) at  $30^\circ\text{C}$  for 30 min, mixed with 0.1 mg of bovine serum albumin and SDS, and heated at  $100^\circ\text{C}$  for 2 min. The products were resolved by continuous 8% polyacrylamide gel electrophoresis. A gel subjected to electrophoresis in parallel and containing untreated Karimabad virus polypeptides gave the expected positions of the Karimabad virus G and N polypeptides as indicated in A and B.

In summary structural analyses of certain of the Phlebotomus fever group viruses have established that they have three unique viral RNA species and three major polypeptides (G1, G2 and N). These analyses confirm the structural similarities to the Bunyavirus genus members (e.g. snowshoe hare virus of the California encephalitis serogroup). However by contrast to all the Bunyavirus genus members so far analysed (reviewed by Bishop & Shope, 1979) whereas the latter have a large  $110\text{--}120 \times 10^3$  dalton G1 and a smaller  $32\text{--}38 \times 10^3$  dalton G2 polypeptides, the Phlebotomus fever group viruses so far analyzed appear to have two glycoproteins of similar size ( $50\text{--}65 \times 10^3$  daltons). However, both virus groups have a similar  $20\text{--}25 \times 10^3$  dalton nucleocapsid protein, N. More data is needed to determine how similar all the Phlebotomus fever group viruses are to each other.

### 3. Oligonucleotide fingerprint and RNA analyses of prototype Dengue viruses.

At the request of WRAIR we have investigated the possibility of distinguishing DEN virus isolates by oligonucleotide fingerprinting techniques. The rationale for these analyses is to determine if it will be eventually possible to (1) determine the origin of the viruses involved in new DEN virus outbreaks and, when a large enough data base is generated, (2) determine whether there are distinguishable virulent DEN strains responsible for hemorrhagic disease symptoms and what is the distribution of these and other DEN virus varieties.

Oligonucleotide fingerprint analyses have been used to characterize and distinguish members of many of the families of RNA viruses, e.g. alphaviruses (Trent *et al.*, 1979, Wengler *et al.*, 1977), arenaviruses (Vezza *et al.*, 1978), bunyaviruses (Clewley *et al.*, 1977a, El Said *et al.*, 1979), orthomyxoviruses (McGeech *et al.*, 1976; Nakajima *et al.*, 1978), picornaviruses (Frisby *et al.*, 1976, Harris *et al.*, 1977), and rhabdoviruses (Clewley *et al.*, 1977b). In general, viruses which can be separated serologically can easily be distinguished by their RNA oligonucleotide fingerprints. In addition many virus isolates which are serologically identical can be distinguished by fingerprinting procedures. Within a virus serotype, as shown for the bunyavirus La Crosse virus (El Said *et al.*, 1979), isolates can be categorized into (a) those with identical fingerprints, or (b) those with comparable, but distinguishable, fingerprints (having few oligonucleotide differences, i.e. genome variants), or (c) those with apparently unrelated fingerprints (genome varieties).

For La Crosse virus, a history of evolution of the virus has been proposed by comparing the fingerprints of the individual virion RNA species of several isolates of the virus obtained at different times and in different places in the midwestern regions of the United States of America (El Said *et al.*, 1979).

Since the question of origin and relatedness of different virus isolates is of importance in documenting the spread of an infectious agent, we have analysed the genome RNA species of prototype strains of the flavivirus Dengue, using comparative oligonucleotide fingerprinting procedures. Our results indicate that there is little, if any, detectable homology between the large, ribonuclease T1 derived, oligonucleotides of the 40 S virion RNA species of prototype DEN 1, 2, 3 and 4 viruses.

Preparations of  $^{32}\text{P}$  labeled DEN viral RNA were resolved by SDS-sucrose gradient centrifugation (Fig. 12), prior to analysis by oligonucleotide fingerprinting. As shown in Fig. 12A, DEN 2 RNA sedimented as a single peak having an approximate S value of 40S (Schlesinger, 1977; Stollar *et al.*, 1966). Identical results were obtained for DEN 1, 3 and 4 viral RNA species. Coelectrophoresis of  $^{32}\text{P}$  DEN 2 RNA within  $^3\text{H}$  uridine labeled snowshoe hare (SSH) RNA (Fig. 12B) indicated that DEN 2 RNA has a molecular weight of  $3.8 \times 10^6$ .

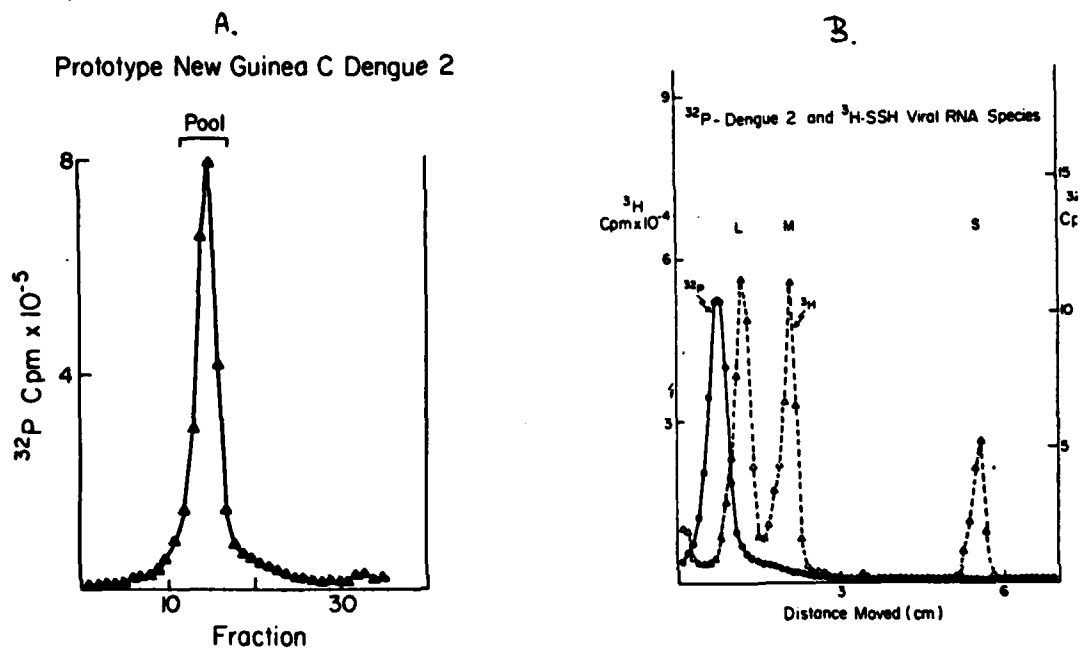


Fig. 12. Resolution of  $^{32}\text{P}$  DEN 2 viral RNA by sucrose gradient centrifugation and (with  $^3\text{H}$  SSH RNA), by polyacrylamide gel electrophoresis.

The base-ratios of the 40 S  $^{32}\text{P}$  labeled viral RNA species (Table 3) obtained from the 4 DEN viruses were found to be similar to each other (Stollar *et al.*, 1966).

Table 3. Base-ratio analyses of the 40 S virion RNA species of prototype Dengue viruses.

<u>Nucleotide</u>	<u>Dengue strain</u>			
	1	2	3	4
CMP	20.7	20.6	20.7	19.6
AMP	31.6	32.5	31.6	30.1
GMP	26.2	25.7	26.4	27.2
UMP	21.5	21.2	21.3	23.1

The base ratios of  $^{32}\text{P}$  labeled 40 S RNA of prototype Dengue viruses were determined as described previously (Clewley et al., 1977a).

Individual preparations of  $^{32}\text{P}$  labeled purified 40 S DEN viral RNA species (0.5 to  $1 \times 10^6$  counts/min) were digested with ribonuclease T1 and the products resolved by two-dimensional polyacrylamide gel electrophoresis as described previously (Clewley et al., 1977 a, b). Shown in Fig. 13 are the fingerprints obtained from four prototype DEN viruses (DEN 1: Hawaii, 1944 isolate; DEN 2: New Guinea C, 1944 isolate; DEN 3: H87, 1956 isolate; and DEN 4 H241, 1956 isolate). Each virus was grown in 5 150 sq cm T flasks at 28°C in MEM for 5 days using cloned *Aedes albopictus* cells and labeled for 2 days at 28°C in MEM containing 200 Ci  $^{32}\text{P}$  per ml, and the virus purified from the supernatant fluids and RNA extracted by standard procedures (Clewley et al., 1977 a, b).



**Fig. 13. Oligonucleotide fingerprints of prototype DEN 1, 2, 3 and 4 40 S viral RNA species.**

In order to determine if there was any extensive oligonucleotide homology between the four prototype DEN viruses, mixtures of the four viral RNA species were digested with ribonuclease T1 and the derived oligonucleotides resolved (Fig. 14, 15).

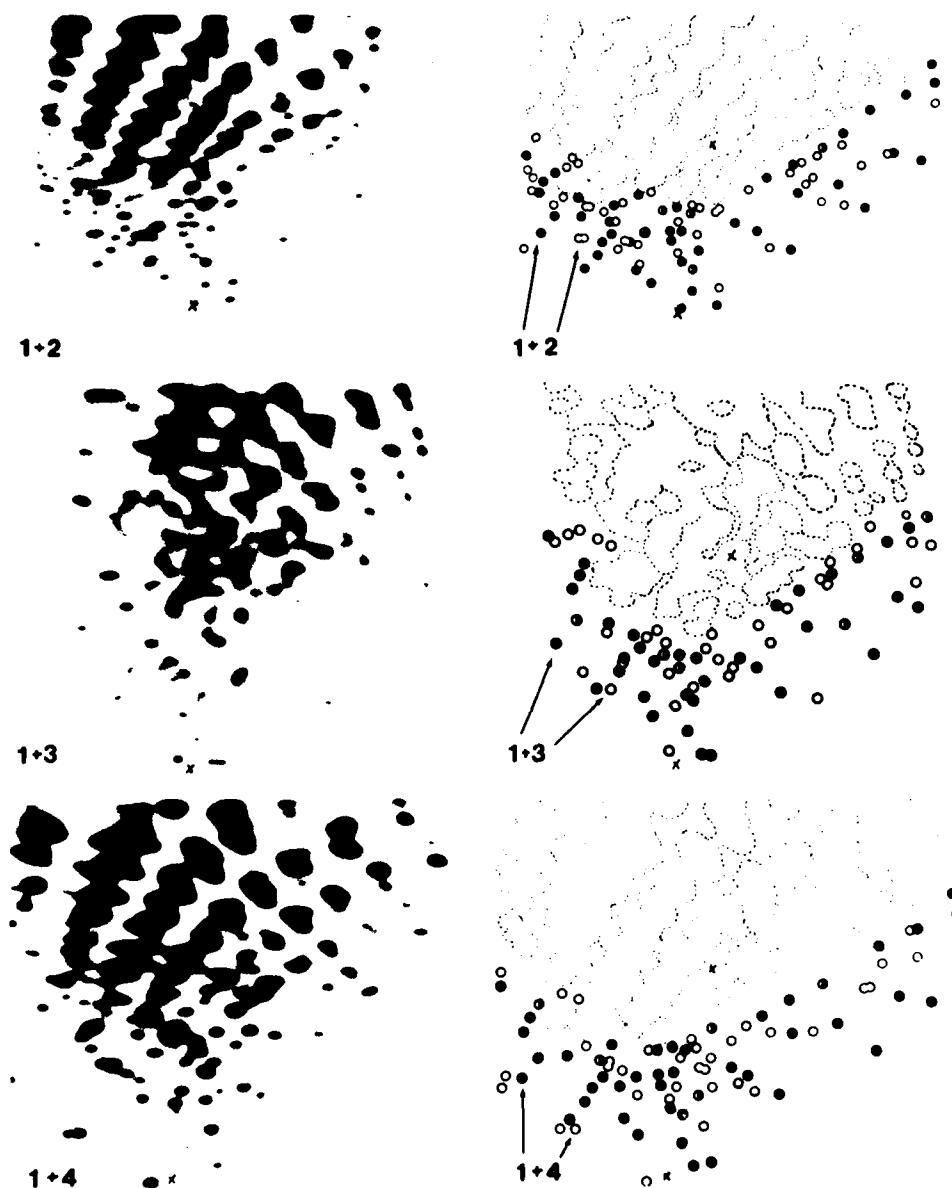


Fig. 14. Oligonucleotide fingerprints (left) and schematics (right) of mixtures of DEN 1 and 2, 1 and 3, and 1 and 4 viral RNA species. The DEN 1 oligonucleotides are indicated by open circles in the schematics and DEN 2, 3 or 4 oligonucleotides by filled circles.

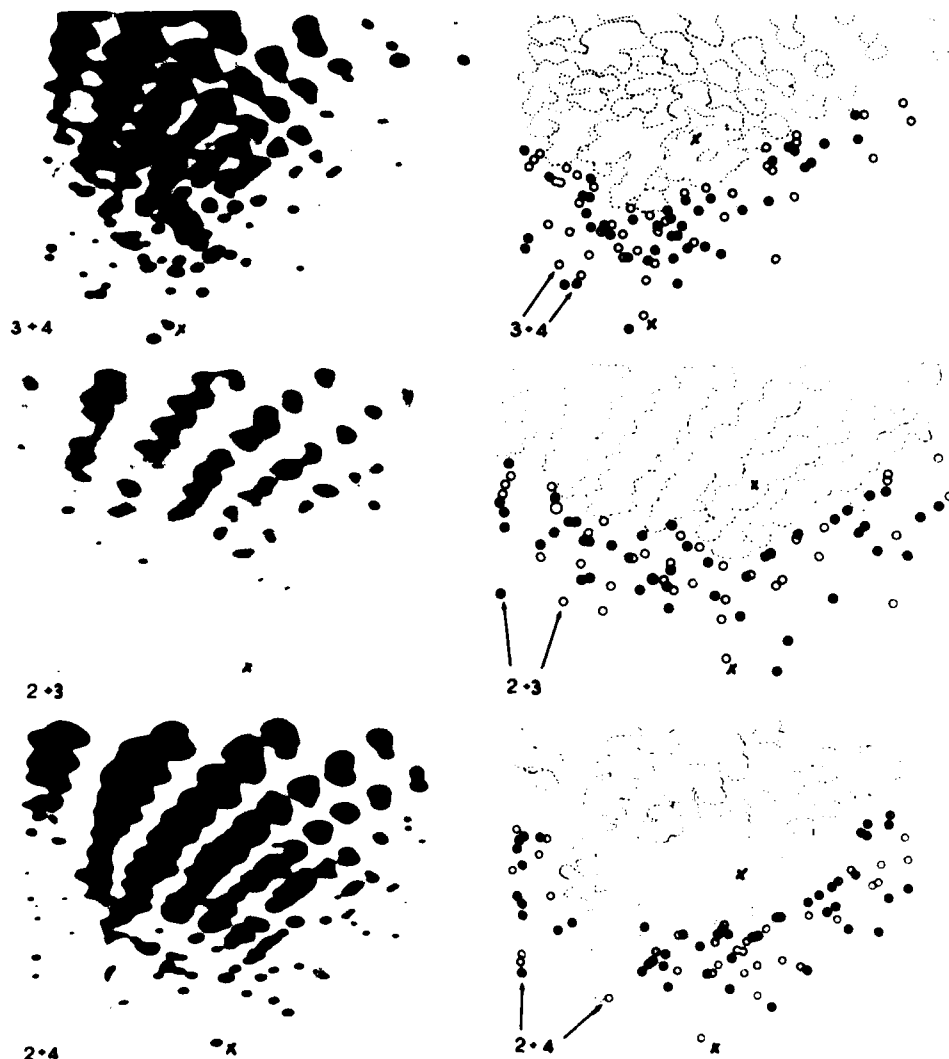


Fig. 15. Oligonucleotide fingerprints (left) and schematics (right) of mixtures of DEN 3 and 4, 2 and 3 and 2 and 4 viral RNA species. The DEN 3 oligonucleotides are indicated in the schematics of the top two panels by open circles (with DEN 4 and 2 oligonucleotides indicated by filled circles). In the bottom schematic panel DEN 4 oligonucleotides are indicated by open circles and DEN 2 oligonucleotides by closed circles.

Although in all of the coelectropherograms the upper half to two thirds of the fingerprints were too complex to analyse (as in the single virus digests -see Fig. 13), the origins of the largest oligonucleotides in the lower portions of the electropherograms were easy to determine by comparing them with the respective single viral RNA digests (see schematics in Fig. 14 and 15). For each viral RNA mixture only a few of the largest oligonucleotides coelectrophoresed (indicated by half filled circles in the right-hand schematics of Fig. 14 and 15). In view of this low number of possibly similar oligonucleotides it was decided that further analyses of each was not warranted since the purpose of these experiments was to determine (1) if the four DEN viral genomes could be distinguished and (2) if there was any evidence of considerable sequence relationship between the genomes of the four DEN serotype viruses.



In order to analyse the 5' terminal sequence of DEN 3 40 S RNA, a preparation of purified  $^{32}\text{P}$  labeled DEN 3 40 S viral RNA was treated with ribonuclease T2 and the digest resolved by DEAE cellulose column chromatography together with optical quantities of marker oligonucleotides from a pancreatic digest of chick embryo RNA. Other than labeled mononucleotides, a single peak of radioactivity was obtained eluting on the leading edge of the marker tetranucleotides (Fig. 16A). When this material was recovered, digested with alkaline phosphatase and rechromatographed, a peak of radioactivity was obtained on the trailing edge of the marker dinucleotides (Fig. 16B). As expected, some  $^{32}\text{P}$  was also recovered in the region of free phosphate. These results are compatible with the 5' terminal sequence of DEN 3 RNA having a cap I type structure i.e.  $\text{m}^7\text{GpppXmpYp}\dots$ , in contrast to the results obtained for the alphavirus Sindbis which has a cap O type structure of  $\text{m}^7\text{GpppApUp}\dots$  (Hefti *et al.*, 1976).

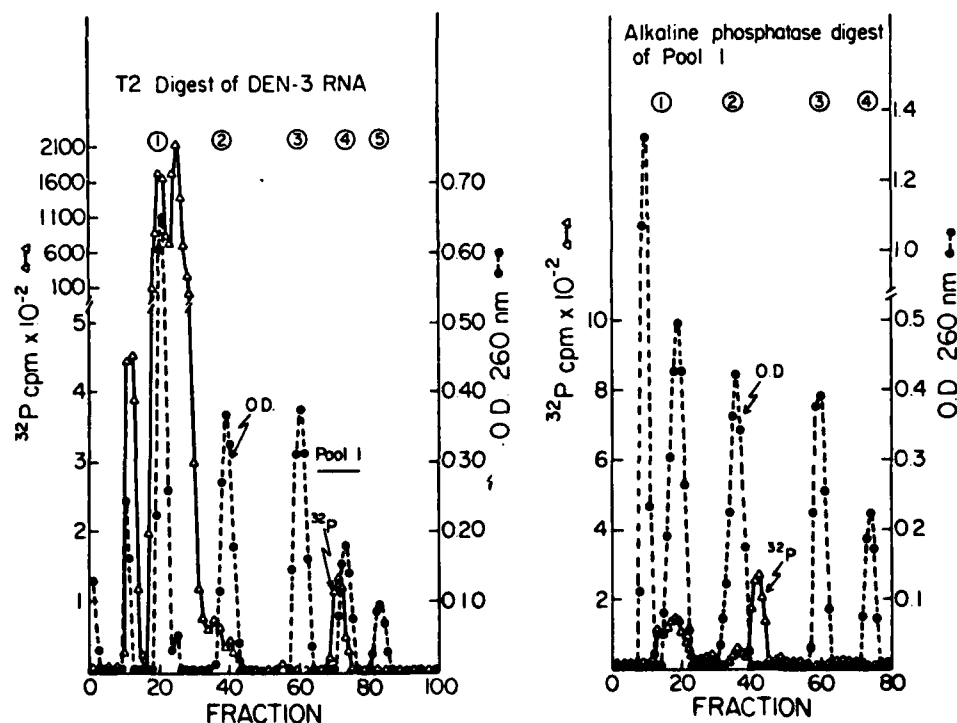


Fig. 16. DEAE cellulose column chromatography of a ribonuclease T2 digest of  $^{32}\text{P}$  labeled DEN 2 RNA coelectrophoresed with optical amounts of a pancreatic ribonuclease digest of chick embryo RNA (left panel). The indicated fragments were pooled, treated with alkaline phosphatase and rerun (right panel) with optical marker oligonucleotides (see Hefti, *et al.*, 1976).

A preparation of (<sup>3</sup>H)methionine labeled DEN 3 40 S RNA was digested with ribonuclease T2 and the products resolved by DEAE cellulose column chromatography. All the radioactivity was recovered in a single peak eluting on the leading edge of the marker tetranucleotides, indicating that (<sup>3</sup>H)methyl labeled nucleotides are only present in the 5' terminal sequences of the viral RNA and that no internal nucleotides are methylated. When another aliquot of the (<sup>3</sup>H)methionine labeled DEN 3 40 S RNA was digested first with ribonuclease T2, then with nuclease P1, pyrophosphatase and finally alkaline phosphatase, and the labelled nucleosides resolved by paper electrophoresis, it was found that essentially equivalent amounts of both m7G and Am nucleosides were present. These results suggested therefore that the 5' cap structure of DEN 3 viral RNA is m7GpppAmpY...

In conclusion the analyses made of 4 prototype DEN virus strains indicate that each is readily distinguishable from another. Since for all four viruses only 10 to 15% of the recovered viral RNA was used for an analysis, it will be easily feasible to fingerprint and compare many different DEN virus strains provided they grow in *Ae. albopictus* cells in amounts comparable to those obtained by us for the 4 prototype strains.

Since these studies were initiated we have trained WRAIR personnel in the required techniques and turned the project over to them for continuation.

#### B. Summary of Progress Report.

The analyses undertaken in the initial 18 months of this contract have established that representative Phlebotomus fever group viruses have a tripartite RNA genome consisting of 3 unique RNA species (L, M and S). Also in parallel to members of the bunyavirus genus, they have a major nucleocapsid protein N and at least for KAR virus (and probably other members of the serogroup), two external unique glycoproteins G1 and G2. However the analyses made to date suggest that the size ranges of the glycoproteins of the Phlebotomus fever group viruses are quite different to those of members of the bunyavirus genus. Interestingly Rift Valley fever virus (another bunyavirus-like virus not assigned to the bunyavirus genus) also appears to have viral polypeptide sizes like those of the Phlebotomus fever group viruses and unlike those of the bunyavirus genus (G. Eddy, personal communication). Detailed analyses of the structural components of other Phlebotomus fever group viruses are planned - see proposal - in order to determine the extent of variation among the structural components of members of the serogroup.

Genetic studies initiated with Punta Toro (PT) virus are incomplete although we have some 24 PT ts mutants and have analysed 5 for recombination capabilities. No evidence for recombination has yet been obtained (not too suprising - see text). Further ts mutant isolation and recombinant tests are planned - see proposal - both for PT virus and other members of the group in order to determine if reassortment of RNA segments can occur and to develop candidate vaccine strains.

Finally at the request of WRAIR we have determined that it is feasible to use oligonucleotide fingerprint analyses to differentiate DEN viruses.

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